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Interpretation of the shape of survival curves in terms of induction and repair/misrepair of DNA double-strand breaks

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Summary Evidence is presented that in yeast cells one DNA double-strand break (dsb) may be considered as one potentially lethal lesion (PLL). Using a temperature conditional radiosensitive diploid yeast mutant (rad 54-3) it is demonstrated that the shoulder of survival curves, for cells plated immediately, is due to repair of dsb (PLL) within a restricted time period. Split dose experiments with the mutant rad 54-3 show that the reappearance of a shoulder is observed when two conditions are met: (1) repair of dsb (PLL) during the time interval between doses and (2) repair of mainly those dsb (PLL) which are induced by the second dose on the nutrient agar plate. Irradiation of wild type yeast cells at high dose rate followed by liquid holding treatment for 72 h (delayed plating, DP) or at low dose rate show that the bending of DP-survival curves is due to the accumulation of dsb (PLL) leading to lethal lesions probably by misrepair of dsb.

The shape of survival curves of eukaryotic cells depends on various physical and biological parameters. The most important physical parameters are LET and dose rate, the most important biological ones are the genetically determined capability of cells to repair critical lesions, time available for this repair and the cell physiological conditions during repair. The eukaryotic yeast provides a system to determine the relative contributions of the physical and biological parameters in shaping survival curves under definite irradiation and post-irradiation conditions. Furthermore, there is a mutant available which allows separation of growth functions and repair functions of the critical lesions.

Using mutants (rad 52) which are deficient in the repair of DNA double strand breaks (dsb) it can be shown that a dsb is a lethal lesion leading to reproductive cell death. Stationary haploid wild type (wt) yeast cells are not able to repair dsb (Luchnik *et al.*, 1977). Their D_0-value of the exponential radiosensitive component of the survival curve equals the D_0-value of the rad 52 haploid yeast (Ho, 1975). Therefore, in diploid wt yeast cells which are proficient in the repair of dsb one dsb may be considered as one potentially lethal lesion (PLL). By using a temperature conditional radiosensitive mutant (rad 54-3) which is able to repair dsb at the permissive temperature of 23°C, but unable to do so at the restrictive temperature of 36°C, it can be demonstrated that the shoulder of survival curves is determined by the restricted repair of dsb. Split dose experiments with this mutant show that the reappearance of a shoulder in a split dose recovery experiment can be explained by repair of dsb (PLL) during the interval between doses *and* on the nutrient agar plate after the second dose. Irradiation of wt yeast cells at high dose rate followed by liquid holding (LH) treatment for 72 h (delayed plating, DP) or at sufficiently low dose rate demonstrates that the bending of DP-survival curves is due to the accumulation of dsb (PLL) leading to cell reproductive death probably by misrepair of dsb.

Materials and methods

Yeast strains

For survival studies three different diploid strains were used: The wt strain 211, the mutant homozygous for the rad 52 gene (Resnick & Martin, 1976) and the temperature conditional radiosensitive diploid yeast mutant rad 54-3 (Budd & Mortimer, 1982) which was generously given to us by Dr J. Game. Its genotype is

$$\frac{\text{rad 54-3} \quad + \quad \text{leu 2} \quad \text{MAT}}{\text{rad 54-3 ade 4 leu 2} \quad \text{MATa}}$$

$$\frac{\text{CAN}^{s}\ \text{ura}^{3} \quad \text{hom 3-10 his 1-7 trp 2}}{\text{CAN}^{r}\ \text{ura}^{3} \quad + \quad \text{his 1-1 trp 2}}$$

In contrast to the wt strain 211 the rad 52 mutant is not capable of repairing dsb. The diploid mutant rad 54-3 is proficient in the repair of dsb at the permissive temperature of 23°C and deficient in dsb repair at the restrictive temperature of 36°C.

For dsb analyses the diploid strain 211*B auxotrophic for 2′-deoxy-thymidine-5′-mono-

phosphate (dTMP) was used which lacks mitochondrial DNA and whose nuclear DNA can be specifically labelled by [^{3}H]dTMP (Höltz & Brendel, 1976). This strain is capable of repairing dsb.

Survival studies and dsb-analyses

For all experiments cells in stationary phase were used. Details of the survival studies with the strain 211, the rad 52-mutant and the rad 54-3 mutant are given elsewhere (Frankenberg, 1979; Frankenberg *et al.*, 1981; Frankenberg-Schwager *et al.*, 1983). For split dose recovery experiments the glucose in the YPD-agar was substituted by sodium succinate (2.7%) which resulted in an increase of shoulder length of the rad 54-3 survival curve relative to glucose. During the split dose interval cells were kept in 67 mmol l^{-1} phosphate buffer, pH = 7.0 (LH—treatment) at the temperature permissive (23°C) or restrictive (36°C) for dsb repair. Details of the dsb analyses are given elsewhere (Frankenberg-Schwager *et al.*, 1979; Frankenberg *et al.*, 1981).

Irradiation

Irradiations were performed with 30 MeV electrons (restricted track average LET, $\bar{L}_{100} = 0.1$ keV μm^{-1}), ^{60}Co-gamma-rays ($\bar{L}_{100} = 0.2$ keV μm^{-1}), 150 kV (or 160 kV) X-rays ($\bar{L}_{100} \approx 2$ keV μm^{-1}), 4 kV X-rays ($\bar{L}_{100} \approx 13$ keV μm^{-1}), 2 kV X-rays ($\bar{L}_{100} \approx 20$ keV μm^{-1}), and 3.5 MeV alpha particles ($\bar{L}_{100} = 65$ keV μm^{-1}) either in suspension or on filters in the presence of oxygen or nitrogen. The irradiations at high dose rate ($\dot{D} \geqq 40$ Gy min^{-1}) were performed at 4°C to avoid dsb-repair during irradiation. This condition is expected to yield optimum accumulation of dsb. During the low dose rate experiments ($\dot{D} \leqq 27$ Gy min^{-1}) cells were kept in phosphate buffer under conditions most favourable for repair of dsb. The dose rates were sufficiently low so that accumulation of dsb during irradiation could not occur.

Results

The dose response curves of the rad 52 mutant after irradiation with 30 MeV electrons, 150 kV, 4 kV and 2 kV X-rays are exponential (results not shown). The survival curves with IP and DP are identical for the rad 52 mutant (Rao *et al.*, 1980). Table I gives the mean initial number of dsb per cell as measured in the strain 211*B per D_0-value of the exponential survival curves of the rad 52 mutant for the different radiations used. The data for 4 kV and 2 kV X-rays are from Binder (1983). It can be seen that for all the radiations used the mean initial number of dsb per cell per D_0 lies between 0.9 and 1.4.

Table I Mean initial number of dsb per cell, $\bar{n}_i$, per D_0-value derived from the exponential survival curves of the mutant rad 52 for different radiations

Radiation	$\bar{n}_i/D_0$
30 MeV electrons, O_2	1.4
30 MeV electrons, N_2	1.3
150 kV X-rays, O_2	0.9
4 kV X-rays, O_2	1.0
2 kV X-rays, O_2	1.4

Using the rad 54-3 mutant it can be demonstrated that the creation of a shouldered survival curve is a consequence of the restricted repair of dsb (Figure 1, top). Rad 54-3 cells plated immediately after irradiation with 30 MeV electrons and incubated at the temperature restrictive for dsb-repair (36°C) yield an exponential survival curve. However, when irradiated cells are plated on nutrient agar and incubated first at the temperature permissive for dsb-repair (23°C) for various periods of time (150 or 330 min) before shifting to the temperature restrictive for dsb-repair (36°C) the fraction of surviving cells increases with increasing time available for dsb-repair on the agar plate. This increased survival is accompanied by the appearance of a shoulder. When irradiated cells plated on nutrient agar are incubated permanently at 23°C the shoulder length is even more pronounced.

A further increase of the shoulder length and increased D_0-values of the exponential part of the rad 54-3 survival curves is observed by DP of cells kept after irradiation under LH conditions for 24, 48 and 72 h at the temperature permissive for dsb-repair before plating cells at 23°C (Figure 1, bottom). In contrast, LH treatment for 72 h at the temperature restrictive for dsb-repair before plating at 36°C yields only a small increase in cell survival (Figure 1, bottom).

The results of split dose experiments with the rad 54-3 mutant are given in Figure 2. This shows the single dose survival curves with IP at the temperature restrictive for dsb-repair (36°C; curve 1) or at the temperature permissive for the repair of dsb (23°C; curve 2). In split dose experiments cells were given a conditioning dose of 200 Gy. During the interval between two doses cells were kept under LH conditions for 72 h at 23°C to allow repair of dsb. After a series of second doses cells were plated immediately on nutrient agar plates either at 36°C to inhibit repair of dsb *on the plate* (curve 3) or at 23°C to allow dsb repair *on the plate* (curve 4). Absence of dsb-repair on the nutrient

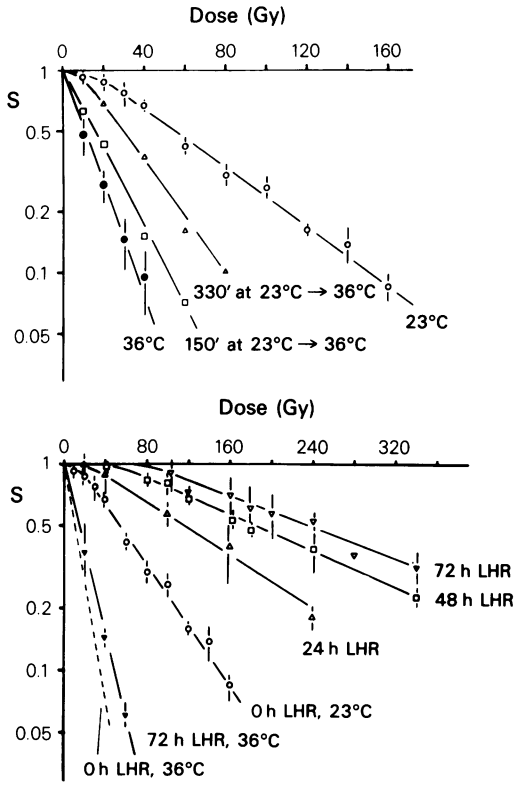

Figure 1 Survival curves of the temperature conditional radiosensitive diploid yeast mutant rad 54-3 under different post-irradiation conditions after irradiation with 30 MeV electrons under oxygenated conditions. For details see **Results.**

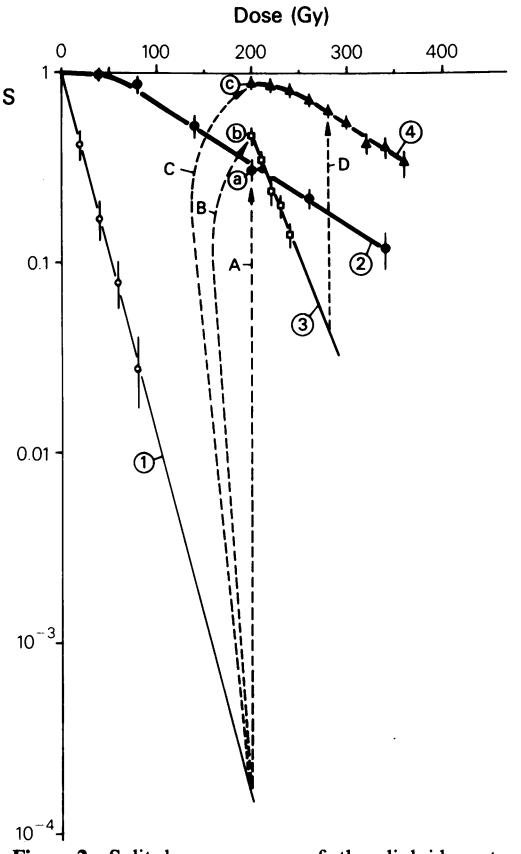

Figure 2 Split-dose recovery of the diploid mutant rad 54-3 and its interpretation in terms of repair of DNA double-strand breaks during the time interval between doses and on the nutrient agar plate after the second dose. Cell platings were performed on YP-sodium succinate agar.

agar plate after the second irradiation yielded an exponential survival curve (curve 3). However, when repair of dsb on the plate was allowed the reappearance of a shouldered survival curve is observed (curve 4). In contrast, when cells are kept during the split dose interval under LH conditions for 72 h at the temperature restrictive for dsb repair (36°C) there was no reappearance of a shouldered survival curve observed after incubation of plated cells at either temperature (results not shown).

Figure 3 (top) shows the survival curves of wt yeast cells obtained after irradiation at low dose rate for ^{60}Co-gamma-rays, 160 kV X-rays, 4 kV X-rays and 3.5 MeV alpha particles. Figure 3 (bottom) shows the corresponding survival curves after irradiation at high dose rate followed by 72 h LH treatment which is sufficiently long to repair all reparable lesions. For technical reasons in the low dose rate experiments ^{60}Co-gamma-rays were used instead of 30 MeV electrons and 160 kV X-rays were used instead of 150 kV X-rays. The DP-survival curves are continuously bent down whereas at very low dose rate exponential survival curves are obtained.

Discussion

Using survival data of the rad 52 mutant and dsb data of the strain 211*B the mean number of dsb per cell per lethal event was determined to be between 0.9 and 1.4 (see Table I). The following consideration shows that it is 1 dsb per cell which corresponds to a lethal event. For 30 MeV electrons the highest amount of energy which can be imparted in a yeast cell nucleus (diameter 1 μm) by one electron is 10 keV. This energy corresponds to 1.6 Gy per cell nucleus. Since a dose of 18 Gy is necessary to induce in the mean 1 dsb per cell (Frankenberg *et al.*, 1981) at least 11 electrons have to transverse the cell nucleus in order to yield this dose. It is therefore fairly improbable that 2 dsb are induced in the DNA by one electron transversal.

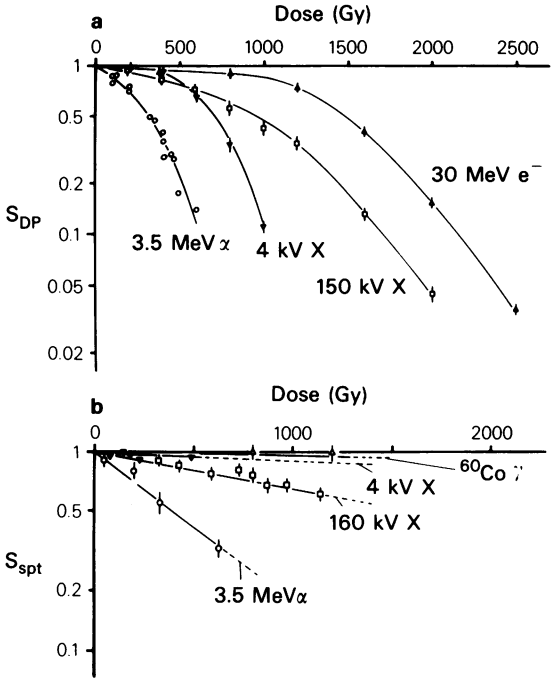

Figure 3 Survival curves of wild type yeast cells at low dose rates (a) and at high dose rates followed by 72 h liquid holding treatment (b).

Table II Dose-independent RBE-values, RBE_{acc}, for the accumulative component S* of DP-survival curves of wild type yeast cells and the RBE-values, RBE_{dsb}, for the induction of dsb for different radiations

Radiation	RBE_{acc}	RBE_{dsb}
30 MeV electrons	1	1
150 kV X-rays	1.3	1.3
4 kV X-rays	2.1	1.8
3.5 MeV alpha part.	3.5	2.6

Thus, if a mean number of 1.4 dsb per cell is needed for one lethal event, a significant fraction of cells has to be inactivated by two electron traversals. The survival curve of the rad 52 mutant would therefore be exponential followed by a shouldered part. However, such survival curves were not observed. A similar consideration can be performed with respect to 2 kV or 4 kV X-rays. Therefore, from the results presented in Table I it is concluded that one unrepaired dsb is lethal, i.e. a dsb is a potentially lethal lesion.

The exponential survival curve of the rad 54-3 mutant cells plated after irradiation at the temperature restrictive for dsb repair (36°C) is converted into a shouldered survival curve when repair of dsb can take place on the nutrient agar plate at the permissive temperature of 23°C (Figure 1, top). The shoulder length can be further increased with increasing amount of dsb repair during LH treatment at 23°C (Figure 1, bottom). These experimental data presented in Figure 1 suggest that the shoulder of survival curves with IP is due to repair of dsb (PLL) which cells can perform between plating on nutrient agar and some unknown point during progression through the cell cycle beyond which no further repair of dsb (PLL) is possible. This is in accordance with theoretical considerations showing that repair of Poisson-distributed dsb (PLL) on the nutrient agar plate within a *restricted* time period yields survival curves with a shoulder (Frankenberg & Frankenberg-Schwager, 1981).

The interpretation of the shoulder of survival curves with IP in terms of repair of dsb (PLL) within a restricted time period suggests that the reappearance of the shoulder in a split dose experiment is due to repair of dsb (PLL) during the interval between two doses *and* on the nutrient agar plate after the second dose. Figure 2 presents the experimental data which support this suggestion. The survival curve 2 – and with it the surviving fraction a at the dose 200 Gy – is due to the repair of dsb (PLL) on the nutrient agar plate after the first dose. The arrow A symbolizes this amount of dsb repair. The amount of dsb repair occurring during the split dose interval (72 h LH treatment at 23°C) (arrow B) can be determined by plating cells after the interval on nutrient agar at 36°C. The corresponding surviving fraction is b. The surviving fraction c is obtained if repair of dsb (PLL) can occur during the interval between two doses *and* on the nutrient agar plate at 23°C (arrow C). Thus, even after a 72 h LH treatment at 23°C some dsb (PLL) are repaired on the nutrient agar plate at 23°C yielding the difference $c-b$ in survival observed after plating at 23°C and 36°C respectively.

If cells are irradiated with a second dose after the interval of 72 h at 23°C during which repair of dsb (PLL) is allowed the exponential survival curve 3 is obtained in the case when dsb repair on the nutrient agar plate is prevented at 36°C. In contrast, the reappearance of a shoulder is observed, if dsb repair is allowed on the nutrient agar plate at 23°C after the second dose (curve 4). The arrow D indicates the repair of dsb on the nutrient agar plate after the second dose. Besides a small amount of dsb due to the first dose mainly those dsb are repaired after plating which are induced by the second dose. As in the single dose survival curve with IP the reappearance of a shoulder is due to the repair of dsb (PLL). In

contrast, when cells are kept during the split dose interval at the temperature restrictive for dsb-repair (36°C) the reappearance of the shoulder was not observed after plating of cells at either temperature (results not shown). Thus, the reappearance of a shoulder in a split-dose experiment is observed when two conditions are met: (1) Repair of dsb (PLL) during the interval between two doses and (2) repair of dsb (PLL) on the nutrient agar plate after the second dose.

Survival curves with IP of wt yeast cells after irradiation at high dose rate comprise both lethal lesions *and* PLL which become lethal under IP-conditions. In contrast, survival curves of wt yeast cells irradiated at high dose rate followed by 72 h LH treatment providing optimum conditions for the repair of PLL (dsb) (DP-survival curves), represent lethal lesions exclusively (Figure 3, top).

The exponential survival curves of wt yeast cells irradiated at very low dose rate reflect the induction of lethal lesions by a single particle traversal (spt) through the cell nucleus (Figure 3, bottom). Comparison of the survival curves at low dose rate and the bent down DP-survival curves obtained at high dose rate suggests that at high dose rate followed by LH treatment for 72 h lethal lesions are produced by accumulation of PLL in addition to those lethal lesions which are produced by a single particle traversal through the cell nucleus at low dose rate. Hence, the DP-survival curves can be described by the equation (1)

$$S_{DP} = S_{spt} \cdot S^* \qquad (1)$$

where S_{DP} is the surviving fraction after irradiation at high dose rate followed by 72 h LH treatment, S_{spt} that after irradiation at low dose rate, and S* is the surviving fraction due to the accumulation effect. In Figure 4 are shown the calculated survival curves S* for the radiations used. By multiplying the doses of 150 kV X-rays, 4 kV X-rays and of 3.5 MeV alpha particles with the corresponding

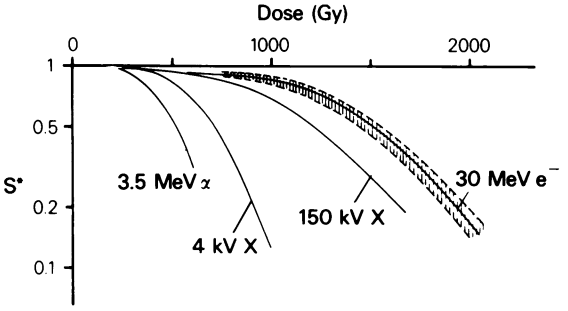

Figure 4 Calculated survival curves S*. Multiplication of the doses of 150 kV X-rays, 4 kV X-rays and 3.5 MeV alpha particles with the corresponding dose-independent RBE_{acc}-values from **Table II** yields S*-curves which fall into the hatched area around the S*-curve for 30 MeV electrons.

dose-independent RBE-values, RBE_{acc}, given in Table II, the S*-curves can be transformed into the S*-curve for 30 MeV electrons. Comparison of these RBE_{acc}-values with the RBE-values for the induction of dsb, RBE_{dsb}, in Table II suggests that in addition to the lethal lesions induced by a single particle traversal through the cell nucleus, lethal lesions are formed at high dose rate due to the accumulation of dsb induced by more than one particle tranversal through the cell nucleus.

In conclusion, data about the induction and repair of dsb allow us to interpret cellular radiobiological phenomena like shoulder length of survival curves, split-dose recovery, and the bending of DP-survival curves after irradiation at high dose rate. This interpretation implicates the DNA as the most important target of ionizing radiations and dsb as the critical lesions for cell reproductive death.

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